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Journal of Invertebrate Pathology 86 (2004) 26–37
Journal of INVERTEBRATE PATHOLOGY
www.elsevier.com/locate/yjipa

Vegetative compatibility groups in indigenous and mass-released strains of the entomopathogenic fungus *Beauveria bassiana*: likelihood of recombination in the field

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Received 30 January 2004; accepted 24 March 2004
Available online 20 April 2004

Abstract

Using nitrate non-utilizing (*nit*) mutants, we determined vegetative compatibility groups (VCG) among strains of *Beauveria bassiana* representing strains indigenous to North America, isolated from diverse insect hosts, and strains that have been mass released for insect control. Genetic similarity among these strains was analyzed using random amplified polymorphic DNA (RAPD) markers. Our data revealed 23 VCGs among the 34 strains tested, with most of these groups comprised of only a single strain. We also observed a VCG comprised of eight genetically similar strains isolated from Colorado potato beetles (CPB). Co-inoculation studies of CPB larvae with complementary *nit* mutants from the same or from different VCGs revealed heterokaryosis in four out of five same-VCG pairs, with only 5–17% of the sporulating cadavers generating few parasexual recombinants. In contrast, none of the infected beetles treated with non-compatible pairs generated recombinants. The large number of VCGs observed and the low frequency of in vivo recombination limited to vegetatively compatible strains indicate that this self/non-self recognition system may be an effective barrier preventing genetic exchange between dissimilar strains in the field.

Keywords: *Beauveria bassiana*; Entomopathogenic fungus; Microbial control; Genetic recombination; Vegetative compatibility

1. Introduction

The fungal pathogen *Beauveria bassiana* is a widely used mycoinsecticide for control of several insect pests, providing a biological alternative to synthetic chemical insecticides (Hajek et al., 2001). A key advantage for microbial control agents is their potential to replicate and persist in the environment, offering continued suppression of insect pest populations. Exploiting this advantage, however, is commensurate with the need to determine the risks to non-target organisms of mass releasing this fungus. To date, no information is available on the potential for genetic recombination between strains of *B. bassiana* in agricultural fields nor on whether this recombination could result in altered virulence and host range.

Genetic recombination in asexual fungi, including *B. bassiana*, can occur through the parasexual cycle, during which vegetatively compatible hyphae fuse to form heterokaryons and exchange genetic material (Bello and Paccola-Meirelles, 1998; Couteaudier and Viaud, 1997; Dalzoto et al., 2003; Paccola-Meirelles and Azevedo, 1991). Compatibility is mediated by multiple vegetative incompatibility loci (*vic* or *het* loci) and is homogenic, requiring identical alleles in all corresponding *vic* loci (Corell et al., 1987). This self/non-self recognition system is hypothesized to restrict transmission of parasitic nuclei or deleterious cytoplasmic elements such as viruses and plasmids (Debets and Griffiths, 1998; Leslie, 1993). Its widespread occurrence further suggests that it is adaptive (Debets and Griffiths, 1998).

Parasexuality has been demonstrated in numerous fungi under laboratory conditions, but its occurrence in nature is difficult to detect. While studies on asexual

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0022-2011/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2004.03.009

fungi have revealed that nearly all fungi examined exhibit recombining population structures in addition to clonal reproduction, current available tests cannot determine how recombination occurs or how often (Taylor et al., 1999). Whether the high level of genetic diversity observed was due to an ancient sexual stage or from more recent (asexual) recombination events, the parasexual cycle is a potential source of gene and genotype diversity in fungal populations (Burdon and Silk, 1997). Furthermore, report of a new pathotype in a fungal plant pathogen as a result of somatic hybridization suggests that genetic exchange during hyphal anastomosis, the initial step in the parasexual cycle, is sufficient in generating altered host range. A study by Park et al. (1999) on the wheat rust fungus *Puccinia recondite* f. sp. *tritici*, reported a new pathotype exhibiting hybrid genotypes of two closely related pathotypes. The new pathotype differed in at least three virulence features indicating that it had arisen not from simple mutational events, but that it was likely a result of recombination.

In this study we identified vegetative compatibility groups (VCG) among strains of *B. bassiana* released as biocontrol agents and naturally occurring strains found in agricultural fields in New York. We wished to assess the likelihood of genetic recombination between *B. bassiana* strains in agricultural fields. We also examined the occurrence of genetic recombination between co-infecting strains of the fungus in a susceptible insect host, the Colorado potato beetle, *Leptinotarsa decemlineata*.

2. Materials and methods

2.1. Fungal strains and culture conditions

Strains of *B. bassiana* from diverse insect hosts collected from various locations in the US and from Quebec and Ontario, Canada, were obtained from the USDA-ARS Entomopathogenic Fungi (ARSEF) Culture Collection in Ithaca, NY, USA (Table 1). These strains represented indigenous populations of *B. bassiana*, including several strains that have been field tested for insect control (Table 2). Also included in this study were *B. bassiana* strains available as mycoinsecticides or were under commercial development as microbial control agents. Strain GHA, the active ingredient in two mycoinsecticides registered in the US for control of several agricultural insect pests, was isolated from a technical product (Lot No. 980528) provided by Emerald BioAgriculture, Lansing, MI. Another strain, ATCC 74040, available as mycoinsecticides (Naturalis products from Troy Bioscience, Phoenix, AZ) was obtained from the American Type Culture Collection. Two strains, NC2 and HF, under development for control of darkling beetles and houseflies, respectively, in poultry houses were provided courtesy of JABB of Carolinas (Pine Level, NC).

Single spore isolates were established for each strain following Veen's protocol (1967), with modifications. A dilute conidial suspension (approximately 10^2 conidia/ml) was prepared for each strain and 0.2-μl droplets was transferred to 25 marked spots on thin plates (~10 ml of medium in a $100 \times 15\,mm^2$ petri dish) of Sabouraud dextrose agar supplemented with 1% yeast extract (SDAY). Plates were incubated at room temperature (~24 °C) for 24–48 h prior to examination under a dissecting microscope at 50× magnification. For each strain, at least five agar blocks ($\sim 2 \times 2\,mm^2$), each with a germinating conidium, were cut using a flamed scalpel and transferred to fresh SDAY plates ($60 \times 15\,mm^2$). Five single spore isolates were established for each strain to assess whether a given strain was mixed or pure. Cultures were grown at room temperature (~24 °C) for 2 weeks prior to sampling conidia or storage at 4 °C. For long term storage, agar blocks ($5 \times 5\,mm^2$) were cut from growing cultures and stored in cryogenic vials with 10% glycerol at −80 °C (Humber, 1997).

2.2. Vegetative compatibility studies

Vegetative compatibility among the different strains of *B. bassiana* was determined by complementation tests between nitrate non-utilizing (*nit*) mutants to visualize heterokaryon formation. To generate *nit* mutants, 100-μl aliquot of spore suspension (10^6 spores/ml of sterile deionized water with 0.2% Tween) was plated on minimal medium (MM) (Bayman and Cotty, 1991) containing 4.5% potassium chlorate (MMCA), or higher at 6% for the more resistant strains. Plates were incubated at room temperature and examined for fast growing colonies after 4–10 days. Ten to twenty chlorate resistant colonies were initially examined to obtain *nit* mutants required for complementation tests. Additional mutants were generated as needed. Putative mutants were transferred to MM plates and colonies that produced sparse growth with no aerial mycelia were considered *nit* mutants. Mutations in the nitrate utilization pathway render these mutants resistant to chlorate, which is reduced to highly toxic chlorite in wild type strains by nitrate reductase (Puhalla, 1985). The *nit* mutants were assigned to one of three phenotypic classes, *nit* 1, *nit* M, or *nit* 3, by their ability to utilize various nitrogen sources (Corell et al., 1987). A 2-mm^3 MM agar block of each mutant was transferred to MM ($60 \times 15\,mm^2$) plates supplemented with ammonium tartrate, hypoxanthine, nitrate or nitrite as sole nitrogen source. Plates were incubated at room temperature (~24 °C) and mutant colony was scored for aerial mycelium (prototrophic growth) after 1–2 weeks.

Self-compatibility tests were initially conducted between *nit* 1 and *nit* M mutants of the same strain prior to

Table 1
Strains of *B. bassiana* investigated in this study

Strain	Host origin	Collection site	Year
GHA	From technical product (Mycotech Industries)		
ARSEF 252	*Leptinotarsa decemlineta* (Coleoptera: Chrysomelidae)	Orono, Maine	1978
ARSEF 317	*L. decemlineata* (Coleoptera: Chrysomelidae)	Rhode Island	1979
ARSEF 344	*L. decemlineata* (Coleoptera: Chrysomelidae)	Yakima, Washington	1979
ARSEF 353	*L. decemlineata* (Coleoptera: Chrysomelidae)	Orono, Maine	1978
ARSEF 533	*O. nubilalis* (Lepidoptera: Pyralidae)	China	1980
ARSEF 1079	*L. decemlineata* (Coleoptera: Chrysomelidae)	Washington	1984
ARSEF 2336	*Schizaphis graminum* (Homoptera: Aphididae)	Parma, Idaho	1986
ARSEF 2430	*Blissus leucopterus* (Hemiptera: Lygaeidae)	Wakefield, Kansas	1980
ARSEF 2580	*Coccinella septempunctata* (Coleoptera: Coccinellidae)	Niles, Michigan	1988
ARSEF 2722	*Solenopsis* (Hymenoptera: Formicidae)	Brazil	1989
ARSEF 2860	*S. graminum* (Homoptera: Aphididae)	Parma, Idaho	1987
ARSEF 2861	*Diuraphis noxia* (Homoptera: Aphididae)	Parma, Idaho	1988
ARSEF 2880	*S. graminum* (Homoptera: Aphididae)	Parma, Idaho	1987
ARSEF 2976	*Ostrinia nubilalis* (Lepidoptera: Pyralidae)	Centre County, Pennsylvania	1990
ARSEF 2987	*L. decemlineata* (Coleoptera: Chrysomelidae)	Quebec, Canada	1988
ARSEF 2989	*L. decemlineata* (Coleoptera: Chrysomelidae)	Quebec, Canada	1988
ARSEF 2990	*L. decemlineata* (Coleoptera: Chrysomelidae)	Quebec, Canada	1988
ARSEF 3111	*Diabrotica virgifera* (Coleoptera: Chrysomelidae)	Mead, Nebraska	1990
ARSEF 3113	Soil → *Ostrinia nubilalis* (Lepidoptera: Pyralidae)	Iowa	1990
ARSEF 3385	*Myzus persicae* (Homoptera: Aphididae)	Yakima, Washington	1991
ARSEF 3386	(Coleoptera: Carabidae)	Yakima, Washington	1991
ARSEF 5339	*Musca domestica* (Diptera: Muscidae)	Seneca County, New York	1989
ARSEF 5345	*M. domestica* (Diptera: Muscidae)	Cayuga County, New York	1990
ARSEF 5493	*Aphis gossypii* (Homoptera: Aphididae)	Pennsylvania	1997
ARSEF 5807	*L. decemlineata* (Coleoptera: Chrysomelidae)	Ontario, Canada	1996
ARSEF 5813	*L. decemlineata* (Coleoptera: Chrysomelidae)	Lansing, Michigan	1998
ARSEF 6148	Soil → *Galleria mellonella* (Lepidoptera: Pyralidae)	Yolo County, California	1999
ARSEF 6986	*L. decemlineata* (Coleoptera: Chrysomelidae)	Freeville, New York	2000
ARSEF 6987	*L. decemlineata* (Coleoptera: Chrysomelidae)	Freeville, New York	2000
ARSEF 6988	*L. decemlineata* (Coleoptera: Chrysomelidae)	Freeville, New York	2000
ATCC 74040	*Anthonomous grandis* (Coleoptera: Curculionidae)	Rio Grande Valley, Texas	1986
NC2	*Alphitobius diaperinus* (Coleoptera: Tenebrionidae)	Greene County, North Carolina	1991
HF	*M. domestica* (Diptera: Muscidae)	Belle, Florida	1997

Table 2
Strains of *B. bassiana* included in this study that have been released or field tested for biological control of insect pests in the US

Strain	Commercial product/target pest	Release site(s)	Reference(s)
GHA	"Botanigard"[a] and "Mycotrol"[a]		
ATCC 74040	"Naturalis"[b]		
ARSEF 252	Colorado potato beetle	Maine, Massachusetts, North Dakota, New Jersey, New York, Rhode Island, Virginia, Washington	Hajek et al. (1987); Gaugler et al. (1989); Poprawski et al. (1997)
	Boll weevil	Texas	Wright and Chandler (1992)
	Pecan weevil	Georgia	Harrison et al. (1993)
ARSEF 533	Corn borer	New york	Feng et al. (1988)
ARSEF 2336[c]	Hop aphid	Idaho	Dorschner et al. (1991)
	Pea aphid	Oregon	James et al. (1995)
ARSEF 2430	Corn rootworm	New York	Kruger and Roberts (1997)
ARSEF 2860	Pear psylla	West Virginia	Puterka (1999)
ARSEF 3113	Corn borer	Iowa	Pingel and Lewis (1996)
ARSEF 3734[c]	Fire ants	Florida	Oi et al. (1994)
ARSEF 5339	Houseflies	New York	Watson et al. (1996)
NC 2	Darkling beetles	North Carolina	Crawford et al. (1998)
HF	Houseflies	North Carolina	(J.J. Arends, pers. communication)

[a] Botanigard and Mycotrol from Emerald BioAgriculture, Lansing, MI, are registered in the US for use against several insect pests attacking a variety of crops.
[b] Naturalis products from Troy Biosciences, Phoenix, AZ, are registered in the US for use against several insect pests attacking a variety of crops.
[c] ARSEF 2336 is identical to ARSEF 2883 and ARSEF 3734 is identical to strain Bb 447 (unpublished data).

their use in inter-isolate pairings. Pairings were conducted by placing a 2-mm^3 MM block of *nit* M mutant in the center of a MM plate ($60 \times 15\,mm^2$), surrounded by four 2-mm^3 MM blocks of *nit* 1 mutants of other strains at a distance of ~1.5 cm. Plates were incubated at room temperature and scored after two weeks or longer for the slower growing strains. *nit* mutants representing all 34 strains were paired in all possible pair-wise combinations. Pairings were replicated at least twice. Strains that showed complementation by forming heterokaryons, evident as a line of aerial mycelium growth at the zone of contact between two complementary mutants were placed in the same VCG. Strains that continued to grow sparsely at their zone of contact were considered vegetatively incompatible.

Following observation of heterokaryons from pairings between *nit* mutants, single spore isolates were established from conidia obtained from the zone of contact. These isolates were cultured on MM plates to ensure prototrophy and to test genetic stability. Single spore isolates were also established from *nit* mutants for further analyses. To obtain conidia for long term storage at −80 °C and for culture of blastospores for DNA extraction, *nit* mutants were grown on potato dextrose agar (PDA) plates at room temperature (~24 °C) for 7–10 days. DNA was extracted from single spore isolates of *nit* mutants and from heterokaryons following the protocol described in Castrillo et al. (2003).

2.3. RAPD-PCR analysis

Genetic similarity among the 34 *B. bassiana* strains was determined by conducting RAPD-PCR assays using twelve 10-nucleotide random primers obtained from Operon Technologies. The 12 primers, OPA4, OPA7, OPA9, OPA11, OPA13, OPB6, OPB10, OPB11, OPC8, OPC9, OPC10, and OPC15, were found to produce robust and reproducible bands in a screening for RAPD primers conducted for another study (Castrillo et al., 2003). A strain of *Metarhizium anisopliae* isolated from Colorado potato beetles from Freeville, NY (ARSEF 7225), was included as an outgroup. Fungal genomic DNA for RAPD assays was isolated as described in Castrillo et al. (2003). RAPD assays were conducted as described in Castrillo et al. (2003). Each RAPD assay was repeated two to three times to ensure reproducibility. Molecular size of PCR products was calculated using TotalLab software for 1D electrophoresis gel analysis (Nonlinear Dynamics, Durham, NC) and the data were analyzed using NTSYS-pc program (Rohlf, 1994). A similarity matrix was calculated using Jaccard's coefficient and clustered using the unweighted pair-group method using arithmetic averages (UPGMA) (Sneath and Sokal, 1973).

To obtain markers for detecting recombination at the molecular level, RAPD assays were also conducted on several *nit* mutants and on heterokaryons obtained from in vitro pairings. These assays were limited to vegetatively compatible strains associated with CPB and primers that generated unique bands among these strains to screen for recombination markers that could be used for co-inoculation studies to follow. RAPD assays on representative wild type parents, their corresponding *nit* 1 and *nit* M mutants, and heterokaryons were conducted using the same PCR conditions as reported above.

2.4. Double-stranded RNA analysis

In addition to RAPD markers, the presence of double-stranded RNA (dsRNA) among the 34 *B. bassiana* strains was evaluated as a potential marker for heterokaryon formation. To detect dsRNA, aliquots of fungal nucleic acids prepared as reported (Castrillo et al., 2003), minus the RNase treatment step, were digested with one of the following nucleases: DNase I (Sigma), which degrades both double- and single-stranded DNA; RNase A (Sigma), which degrades both double- and single-stranded RNA; or S1 nuclease (Invitrogen), which degrades single-stranded DNA or RNA. Reaction conditions were as described in Sambrook et al. (1989). An aliquot of each digestion mix for each strain was run in a 1% agarose gel stained with ethidium bromide to visualize the results. Lambda phage DNA digested with restriction endonucleases *Eco*R1 and *Hin*dIII (Invitrogen) was used as a molecular size marker. Nucleic acids resistant to DNase 1 and S1 nuclease were considered to be dsRNA. Following screening among the wild type parent strains, the presence of dsRNA was determined in corresponding *nit* mutants of strains with dsRNA and in heterokaryons formed between pairs with one or both parents containing dsRNA.

2.5. Co-inoculation studies

To determine the likelihood of recombination in the field, laboratory-reared third instar CPB larvae were co-inoculated with complementary *nit* mutants from the same or different VCGs. A preliminary assay was conducted first using single inoculations of either 5813 *nit* 1 or *nit* M mutants to test for frequency of reversion to prototrophy, which would complicate scoring for heterokaryons in co-inoculated larvae. Additional preliminary assays were done using pairs of complementary *nit* mutants (5813 *nit* 1 + 5813 *nit* M, 252 *nit* M, 5807 *nit* M, 74040 *nit* M, GHA *nit* M, or 6986 *nit* M and 5807 *nit* 1 + 5813 *nit* M) to develop handling and sampling protocols and to test whether *nit* mutants were pathogenic to CPB larvae. For each treatment 20 insects were inoculated using a spray tower following the method of Vandenberg (1996). A single or combined dosage of

approximately 580 spores/mm^2, estimated to give 50% mortality (unpublished data), was applied. Conidia for the bioassay study were obtained from 14-day cultures of *nit* mutants and wild type strains on plates of PDA incubated at room temperature (~24 °C). Conidia were brushed into sterile 50-ml conical polypropylene tubes, using autoclaved 18-mm camel-hair brushes, and dried overnight in a dessicator before storage at −20 °C. Viability of spores and dosage estimation were determined according to the method of Vandenberg (1996). Following spray treatment, each test insect was transferred to a sterile petri dish ($60 \times 15\,mm^2$) lined with moist filter paper and provided with potato foliage for food. Mortality was recorded daily. Dead insects were transferred to sterile Petri dishes ($100 \times 15\,mm^2$) lined with moist filter paper and observed for fungal sporulation. Spore progeny from representative infected cadavers were sampled using a flamed inoculating loop and suspended in sterile aqueous Tween solution (0.2%) to obtain a concentration of approximately 10^2–10^3 spores/ml. Aliquots of 100 µl were plated on MM to detect presence of recombinants from *nit* pairings or revertants from single *nit* mutant treatments. MM plates were kept at room temperature and scored for prototrophic growth after 3–7 days.

Following the preliminary study, co-inoculation studies were done on third instar CPB larvae using pairs of *nit* mutants, along with pairs of their wild type parent strains to determine changes, if any, in virulence of *nit* mutants. For each treatment 3 replicates of 30 larvae were sprayed and handled following protocols used in the preliminary study. For this study, sporulated cadavers were individually suspended in 10 ml of sterile aqueous Tween solution (0.2%) and vortexed for 5 min. A serial dilution was prepared and 100-µl aliquots were plated on MM, MM + hypoxanthine, and PDA to screen for parasexual recombinants and to provide an estimate of the proportion of *nit* 1 and *nit* M mutants and total CFUs per plate for a given dilution, respectively. These media were supplemented with antibiotics (70 mg penicillin G and 100 mg streptomycin sulfate/liter of medium) to minimize contamination with bacteria from cadavers. Two to three plates of each medium were inoculated per dilution per sample insect. Plates were incubated at room temperature (~24 °C) and scored after 5–10 days. Putative recombinants, visible as CFUs with aerial mycelia and spore formation on MM, were sampled using a flamed inoculating needle and transferred to fresh MM plates ($60 \times 15\,mm^2$) to ensure prototrophy. Single spore isolates were established from representative recombinants for molecular analysis using RAPD and dsRNA markers.

To test whether the absence of parasexual recombinants observed in treatments with vegetatively compatible strains was due to low frequency of recombination events, an additional test was conducted using pairs 5813 *nit* 1 + 317 *nit* M and 6986 *nit* 1 + 317 *nit* M with 2 replicates of 30 larvae each. Test insects were handled as described above.

3. Results

3.1. Vegetative compatibility groups

Fast growing CFUs were observed on MM with 4.5% chlorate after 5–7 days for most of the *B. bassiana* strains investigated in this study. Several strains were more resistant to chlorate and required MM with 6% chlorate to generate *nit* mutants. Phenotype screening of *nit* mutants took about 2 weeks to obtain good comparison of growth among cultures on different nitrogen sources. Most of the *nit* mutants obtained were identified as *nit* 1. Generally, 20 *nit* mutants need to be generated to obtain 1–2 *nit* M mutants. *Nit* 3 mutants were rare and observed from only 3 strains (1079, 3386, and 6988). Complementary tests between different strains were, thus, limited to those between *nit 1* and *nit* M mutants. Intra-isolate pairings of *nit* 1 and *nit* M mutants showed all strains to be self-compatible.

Compatible reactions, resulting in heterokaryon formation, varied in strength based on growth characteristics of aerial mycelium formed at points of contact between two complementary *nit* mutants. Some pairs produced dense and profuse mycelial growth, followed by production of numerous conidia, while a few pairs produced a thin line of aerial mycelia that took up to 6 weeks to sporulate. This variation in the morphology of heterokaryons was also observed for some strains in the self-compatibility tests.

Among the 34 *B. bassiana* strains tested, 23 VCGs were observed with most of these groups comprised of only a single strain (Fig. 1). Three VCGs, groups 2, 5, and 12, were comprised of 2–8 strains. VCG 2 was made up of 8 strains, most of which were isolated from CPBs collected from northeastern North America over a period of more than 20 years (Fig. 2). One strain in this group, 2985, was isolated from a curculionid from Quebec, Canada. VCG 5 was made up of 2 strains from houseflies from poultry houses from 2 different counties in New York collected a year apart. VCG 12 included 4 strains collected from aphids from Parma, ID, over a period of 3 years.

Among the 12 *B. bassiana* strains included in this study that have been commercialized as mycoinsecticides or field tested for insect control, two strains (74040 and 252) belonged to VCG 2 (Fig. 2A), 2 (2336 and 2860) belonged to VCG 12, and one strain (5339) belonged to VCG 5. The other 7 strains, including GHA, were incompatible with all of the other strains tested (Fig. 2B).

3.2. Genetic similarity

Molecular analysis of the 34 *B. bassiana* strains and one *M. anisopliae* strain against 12 RAPD primers generated 205 bands resolving the different fungal strains into 32 genotypes (Fig. 1). Two pairs of *B. bassiana* strains were found to be identical: strains 2860 and 2861 collected from two different species of aphids from Idaho from two consecutive years, and strains 252 and 317 collected from CPBs from Maine and Rhode Island, respectively, also from two consecutive years. For the rest of the *B. bassiana* strains assayed, genetic similarity ranged from 13 to 96%.

Genetic similarity among strains collected from the same location and/or host was variable. For example, 3 distinct genotypes observed among strains collected from CPB from the same field and sampling date in Freeville, NY, shared only 34% genetic similarity. In contrast, a high level of genetic similarity, 73%, was observed among strains collected from aphids from Parma, Idaho.

Correlation of RAPD data with VCG showed that most members of the same VCG shared a high level of

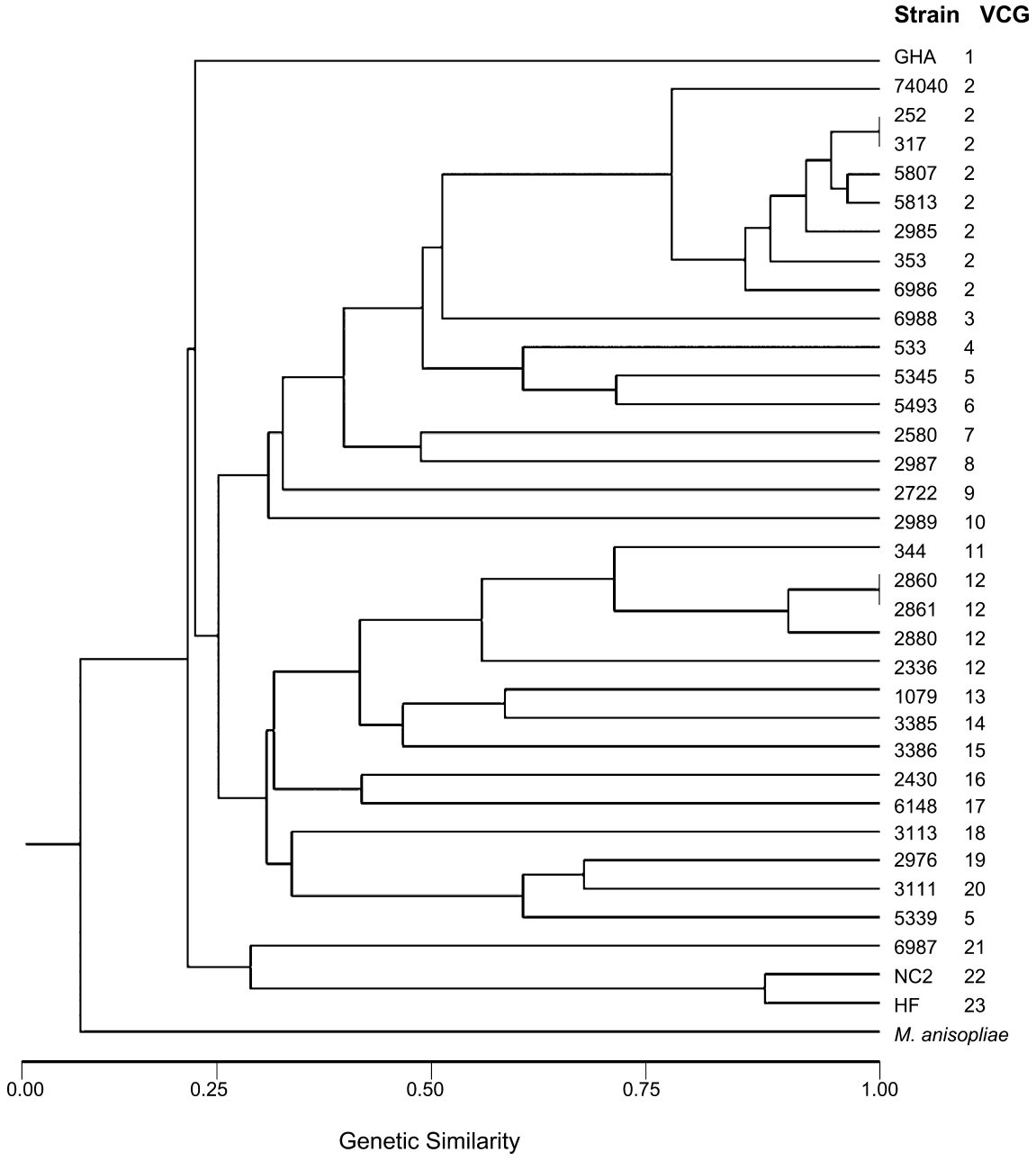

Fig. 1. Dendrogram representing genetic similarity based on Jaccard coefficient among 34 strains of *B. bassiana* and one strain of *M. anisopliae*. Similarity coefficients were based on 205 RAPD markers amplified with 12 primers. Cluster analysis was performed using UPGMA. Vegetative compatibility groups (VCG) are indicated in the right hand column.

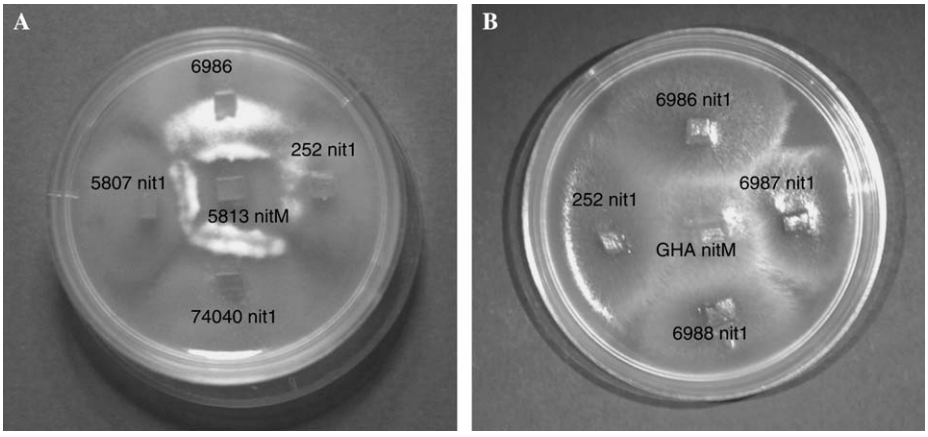

Fig. 2. Complementary pairings of *nit* M (center block) and *nit* 1 mutants (surrounding blocks) derived from strains of *B. bassiana*. Compatibility is scored by the presence (A) or absence (B) of aerial mycelia at zones of contact between *nit* 1 and a *nit* M mutants. Strains 252, 5807, 5813, 6986, and 74040 are compatible and belong to one vegetative compatibility group, while strain GHA is incompatible with strains 252, 6986, 6987, and 6988.

genetic similarity (Fig. 1). VCG 2 and 12 were made up of strains sharing 86% (range of 68–96%) and 73% (range of 49–100%) similarity, respectively. However, the two strains in VCG 5 only shared 33% similarity. Furthermore, not all strains sharing high level of genetic similarity were vegetatively compatible. For example, NC 2 and HF shared 86% similarity but were incompatible. Also, strain 344, from a CPB collected from Washington, was genetically more similar to strains 2860, 2861, and 2880 than strain 2336, 69% versus 53% similarity, but was of a different VCG. The latter four strains comprised VCG 12.

3.3. Recombination markers: RAPD markers

RAPD assays of *nit* 1 and *nit* M mutants from strains belonging to VCG 2 revealed RAPD patterns identical to their parent strain. Assays of heterokaryons obtained from complementation tests on MM showed hybrid patterns from the two parent strains in cases where the primer tested generated unique bands in one parent. For example, primer OPB-10, which generates a unique 1.45 kb product in 5813 and both of its *nit* mutants, also generated the same product in heterokaryons formed between 5813 *nit* 1 + 252 *nit* M and 5813 *nit* M and 5807 *nit* 1 (Fig. 3). None of the heterokaryons from any of the pairings among strains in VCG 8 were found to exhibit novel banding patterns.

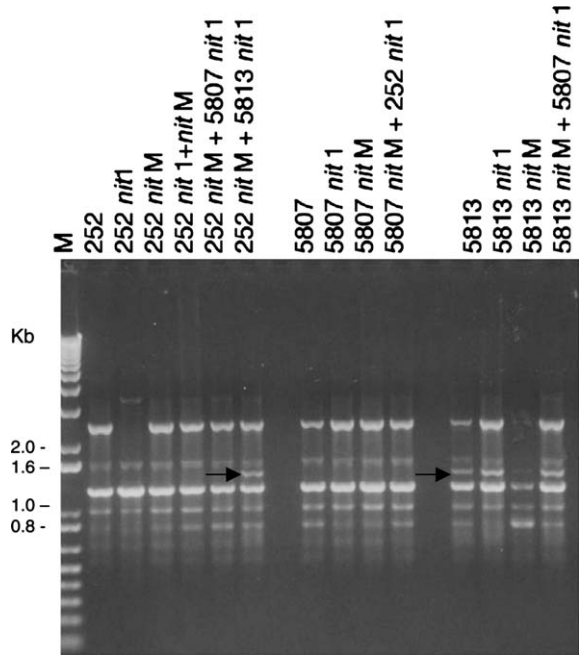

Fig. 3. RAPD analysis of three *B. bassiana* wild type strains, *nit* 1 and *nit* M mutants derived from each strain, and heterokaryons formed between complementary *nit* mutants by use of primer OPB-10. This primer generates a 1.45 kb product (see arrow) unique to strain 5813, which was used as a marker for in vitro heterokaryons formed between 5813 and compatible strains 252 and 5807. M is molecular marker 1 kb plus (Invitrogen).

3.4. Recombination markers: dsRNA

Double-stranded RNA was found in 7 of the *B. bassiana* strains in the study. Four of these strains (74040, 317, 2985, and 6986) belong to VCG 2. The number of dsRNA present among these strains varied from 2 to 5. Comparison of dsRNA patterns among these 4 strains and their corresponding *nit* mutants revealed the loss of dsRNA following selection on chlorate medium for most of these strains. Only 6986 *nit* 1 and 317 *nit* M still had dsRNA (Fig. 5). In the 6986 *nit* 1 mutant only one dsRNA was present compared to the 5 observed in the wild type parent (Fig. 5). Strain 317 and its *nit* M mutant had 3 dsRNA (Fig. 5). Heterokaryons sampled from in vitro pairings between 6986 *nit* 1 and 252, 5807 or 5813 *nit* M revealed presence of dsRNA of similar size to those observed in 6986 *nit* mutant.

3.5. Co-inoculation studies

Preliminary studies revealed that *nit* mutants were pathogenic to third instar CPB larvae, causing 25–35% mortality in treatments with either 5813 *nit* 1 or *nit* M mutant, respectively, and 20–60% mortality in treatments with a combination *nit* 1 and *nit* M mutants. Plating of spore progeny from single *nit* treatments did not reveal any prototrophic growth or revertants. In contrast, heterokaryons were observed among spore progeny from two of the combination treatments, 5813 *nit* 1 + 74040 *nit* M and 5807 *nit* 1 + 5813 *nit* M.

In the co-inoculation study, treatment of CPB larvae with approximately 580 spores/mm^2 of a combination of 2 wild type parents or *nit* 1 and *nit* M mutants resulted in 43–72% and 47–71% mortality, respectively. The total number of insects sampled per treatment and the number from which parasexual recombinants were obtained are listed in Table 3. Among treatments with compatible *nit* pairs, 4 out of 5 resulted in heterokaryon formation, exhibited as prototrophic growth on MM of some spore progeny. In contrast, neither treatments utilizing GHA, which was of a VCG by itself, resulted in CFUs with prototrophic growth.

Among compatible *nit* treatments that resulted in CFUs with prototrophic growth, only 5–17% of cadavers examined generated recombinants. Examination of MM + hypoxanthine plates also revealed that although an equal mix of complimentary *nit* mutants was used in the inocula, spore progeny from cadavers showed a variable proportion of each *nit* mutant. While most cadavers produced approximately equal *nit* 1 and *nit* M mutant spore progeny, a few produced predominantly one of either *nit* mutant. The number of recombinants observed from a given cadaver was also low, <1–3%, and most were observed from plates inoculated with concentrations of 10^3 or more spores. Comparison of MM plates on which recombinants were detected versus plates of MM + hypoxanthine revealed that in cadavers from which recombinants were observed approximately equal numbers of *nit* 1 and *nit* M mutants were present among the spore progeny.

Table 3
Pairs of complimentary *nit* mutants used to treat Colorado potato beetle larvae for in vivo recombination study

Treatments	No. of CPB sampled[a]	No. with parasexual recombinants
(*nit* 1 + *nit* M mutants)		
5813 + 252	21	1
5813 + 6986	20	3
5813 + GHA	18	0
6986 + GHA	16	0
5813 + 74040	27	0
5813 + 317		
(Test 1)	28	0
(Test 2)	30	10
6986 + 317		
(Test 1)	24	0
(Test 2)	40	5

[a] A total of 90 CPB larvae were initially sprayed with approximately 580 spores/mm^2 of *nit* mutants to obtain approximately 50% mortality. Parasexual recombinants present among spore progeny were initially detected by the presence of CFUs with dense aerial mycelia on MM inoculated with spore suspensions from infected cadavers.

Although treatments including GHA *nit* M resulted in 62–71% mortality, fewer samples were analyzed because of poor sporulation observed among these cadavers. We found mortality among test insects in treatments with either GHA wild type or *nit* M mutant to commence a day or two early, days 3–4 after spraying, than in the other treatments. These larvae that died earlier resulted in smaller cadavers producing sparse mycelial growth and consequently, fewer conidia for sampling. In contrast, most of the mortality in the other treatments were observed on days 6–7 after spraying, resulting in bigger cadavers producing dense mycelial growth and numerous conidia.

Proportion of *nit* 1 and *nit* M mutants was estimated only in terms of CFUs on either MM or MM + hypoxanthine plates. Because CFUs may have originated from more than one conidium, estimates of recombination among quantified conidia were not made. Observations were limited to 7 days following plating because in vitro heterokaryon formation began to occur. These heterokaryons differed from the recombinant spore progeny formed inside the larvae by their longitudinal growth along zone of contact between compatible mutants.

Molecular analyses of representative recombinants from CPB larvae utilizing RAPD markers supported the results of screening on MM for putative parasexual recombinants. Recombinants generated hybrid banding patterns representing the two parental genotypes in assays with different primers. For example, pairing of 5813 *nit* 1 and 6986 1 *nit* M mutants produced heterokaryons with a 1.45 kb band, which is unique to 5813, in assays with primer OPB-10. And in assays with primer OPA-13, these heterokaryons produced 1.0 and ~1.3 kb bands, which are unique to 6986 (Fig. 4). In pairings with one parent possessing a dsRNA, some heterokaryons were observed to exhibit dsRNA of similar size as the donor parent. In pairing 6986 *nit* 1 and 317 *nit* M, both of which had dsRNA, heterokaryons were found to contain variable numbers of dsRNA. In some samples, the number of dsRNA observed was more than the total numbers combined from the two *nit* mutants and reflected dsRNA patterns similar to the wild type parents (Fig. 5).

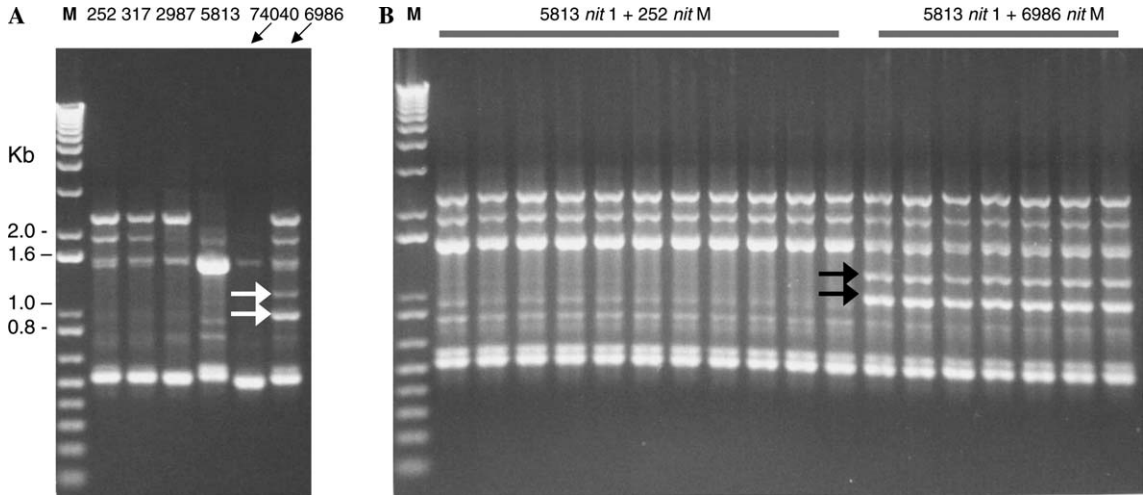

Fig. 4. RAPD analysis of six vegetatively compatible *B. bassiana* strains (A) and recombinants (B) obtained from Colorado potato beetles co-inoculated with 5813 *nit* 1 and 252 *nit* M or 5813 *nit* 1 and 6986 *nit* M mutants. Primer OPA-13 generates two unique bands, 1.0 and ~1.3 kb, in strain 6986 (see large arrows), which were used as markers for recombinants formed between 5813 *nit* 1 and 6986 *nit* M mutants. M is molecular marker 1 kb plus (Invitrogen).

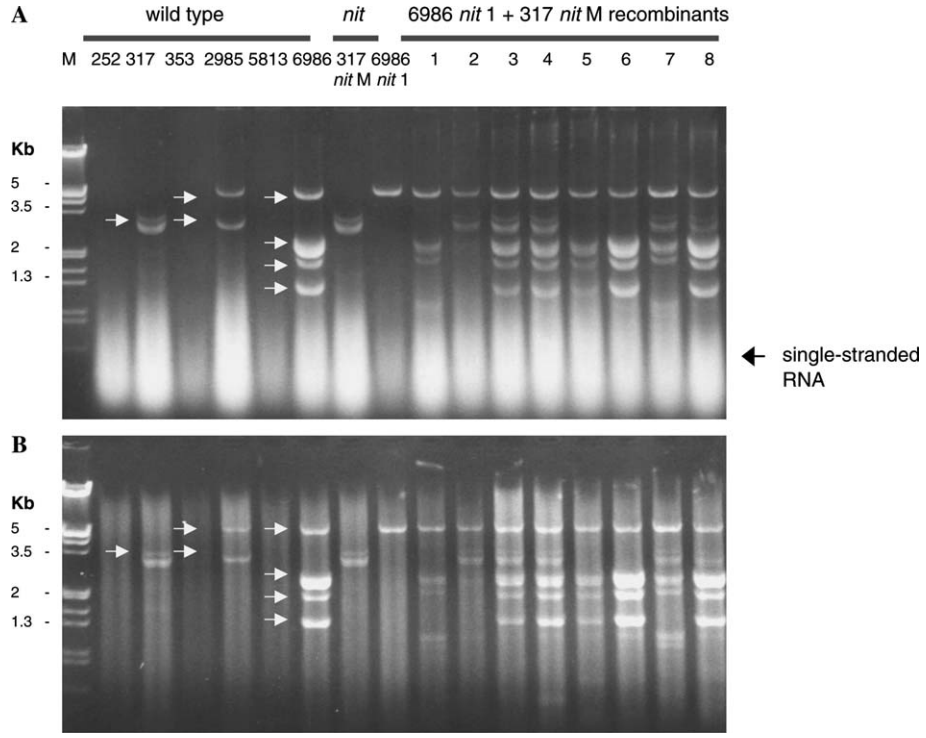

Fig. 5. Double-stranded RNA (dsRNA) was detected in *B. bassiana* strains by digesting total nucleic acid extracts with DNase 1(A) or S1 nuclease (B). dsRNA (white arrows) was found in wild type strains 317, 2985, and 6986 from vegetative compatibility group 2. Among *nit* mutants generated from these strains, dsRNA was retained in 6986 *nit* 1 and 317 *nit* M mutants. Heterokaryons formed between these *nit* mutants displayed dsRNA patterns reflecting mixing of the two *nit* parents. M is Lambda DNA/*Eco*RI + *Hin*dIII marker (Promega).

4. Discussion

Strains of *B. bassiana* collected from various insect hosts from different parts of North America and released in the field as mycoinsectides showed a high level of genetic diversity as evidenced by resolution of the 34 strains into 32 separate genotypes and into 23 VCGs. Similarly, a study by Couteaudier and Viaud (1997) on VCG groups in 25 strains of *B. bassiana* from different insect hosts collected from different countries reported 14 VCGs corroborating our observation of high polymorphism in the *vic* loci.

Among the 34 strains, only three VCG were comprised of two or more members. Genetic analyses with RAPD markers showed high genetic similarity among most strains within a VCG. In VCG 12, comprised of four isolates from aphids, three strains were near clonal, and in VCG 2, comprised mostly of strains associated with CPBs collected from different parts of the country and from Canada, 86% similarity was shared. In most of the strains from either VCG 2 or 12, distinct RAPD banding patterns were evident correlated to either VCG. Couteaudier and Viaud (1997) also found correlation between the number of VCGs observed in *B. bassiana* with high level of variations in telomeric patterns suggesting limited gene flow resulting in genetically diverse populations.

While most studies on compatibility in asexual fungi have shown genetically similar isolates comprising a VCG, a few studies have shown evidence to the contrary (Cantone and Vandenberg, 1998; Chulze et al., 2000; Jacobson et al., 1993). In the ectomycorrhizal fungus *Suillus granulatus*, Jacobson et al. (1993) found that while compatible strains have high genetic similarity based on RAPD marker analysis, a few were genetically distinct sharing no more than 39% similarity. Likewise, we found 2 compatible strains with only 33% similarity. Assuming that compatibility is homogenic and is based on multiple alleles in *B. bassiana*, a compatible reaction observed in vitro may be due to the stressful conditions to which these fungal strains were subjected. Parasexual recombination between incompatible strains of the same species and between closely related species was found to be prevalent under stressful environmental conditions (Julian et al., 1999; Molnar et al., 1990).

Genetic exchange between incompatible fungi has also been documented in fungal strains via horizontal transfer of dsRNA (Rosewich and Kistler, 2000). Many fungi contain cytoplasmic viruses that generally have dsRNA genomes. Transmission of these dsRNA between fungal strains occurs in connection with hyphal anastomosis and is common when hyphae are compatible. Between incompatible strains, transmission efficiency decreases with an increasing number of *vic* genes (Liu and Milgroom, 1996). The variable transmission rate of dsRNA between incompatible strains limits the use of dsRNA as recombination markers for the parasexual cycle. However, their transmission between vegetatively compatible strains in our study provided additional evidence of hyphal anastomosis resulting in the transfer of cytoplasmic elements. Furthermore, dsRNA could be used to examine the extent to which incompatible pairs could still anastomose and exchange cytoplasmic material.

Although parasexuality has been documented in a wide range of asexual fungi under laboratory conditions, its biological significance in the field in poorly known. The frequency of this process in nature is difficult to establish because of the absence of strain-specific markers. By assessing genetic diversity and VCG among indigenous and commercial strains of *B. bassiana*, we are taking the initial steps in evaluating the potential for recombination between strains present in the field. Under field conditions in which endemic infections of a pest by *B. bassiana* occur, inundative application of another strain makes co-infections possible or even likely. Close proximity within the confines of the host hemocoel increases the chance of hyphal contact and anastomoses between compatible strains.

In the co-inoculation studies of CPB larvae with complementary *nit* mutants, the relevance of in vitro VCG data to potential field situations was tested. Although correlation between in vitro and co-inoculation studies was limited given the different conditions under which the two *nit* mutants co-exist and the complexity of interactions involved between fungus and insect host during the infection process, results of our in vivo tests supported results obtained in vitro. Parasexual recombinants were observed only among spore progeny from treatments with compatible *nit* mutants. The frequency of recombination observed was higher than the frequency, 10^{-3}–10^{-7}, estimated by Caten (1981) based on in vitro data, but lower than those reported in co-inoculation studies with *Paecilomyces fumosoroseus*. Riba and Ravelojoana (1984) reported close to 90% heterokaryosis in a study using diauxotrophic mutants of *P. fumosoroseus*. Co-inoculations studies conducted by Leal-Bertioli et al. (2000) and Wang et al. (2002) reported recombination frequency of 10–33% in *M. anisopliae* and 43% in *B. bassiana*, respectively, using wild type strains. The high frequency of recombination observed in these two studies is surprising given their use of wild type strains and the small number of spore progeny they examined. Correlation of their results with ours, however, is difficult because of the absence of information on VCG among strains they used. For recombination events that are relatively rare, the use of auxotrophs provides a way to screen numerous spore progeny, as well as provide initial evidence of recombination.

Absence of recombination between *B. bassiana* strain GHA and the other strains in co-inoculation studies does not completely preclude genetic exchange between this strain and others. Critical in the conduct of co-inoculation studies are the selection of the right combination dose and spray method to optimize infection with both strains. For a strain like GHA, which killed *L. decemlineata* larvae more rapidly than other strains, equal numbers of conidia applied at the same time still resulted in earlier mortality compared to treatments without GHA. Smaller cadavers with fewer conidia were observed on cadavers from treatments with GHA and, consequently, led to a smaller sample size to study. Analysis of spore progeny from these cadavers showed

mostly GHA *nit* mutants. Although the potential for recombination between this mass-released strain and incompatible indigenous isolates cannot be excluded, the low recombination frequency observed in vivo among compatible treatments suggests that recombination events between incompatible strains would be rare.

In agricultural ecosystems where inundative quantities of *B. bassiana* are regularly released, the likelihood of recombination between commercial and indigenous strains needs to be accurately assessed. This can lead to an evaluation of the benefits of using this microbial control agent against any risks, real or perceived, of its use. These factors may then be incorporated into larger framework for making pest management decisions.

Acknowledgments

We thank Richard Humber and Karen Hansen (USDA-ARS, Ithaca, NY) for providing fungal isolates from the ARSEF collection, Emerald BioAgriculture Corp. (Lansing, MI) for providing strain GHA, and Jim Arends (JABB of the Carolinas, Pine Level, NC) for providing strains NC2 and HF. This paper reports the results of research only. Mention of a proprietary product does not constitute a recommendation or endorsement by the US Department of Agriculture for its use.

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